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Prokineticins (Endocrine Gland-Derived Vascular Endothelial Growth Factor and BV8) in the Bovine Ovary: Expression and Role as Mitogens and Survival Factors for Corpus Luteum-Derived Endothelial Cells

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A highly vascular endocrine gland, the corpus luteum (CL) is an excellent model for the study of angiogenic factors. Prokineticins (PK-1 and -2), also termed endocrine-gland-derived vascular endothelial growth factor (VEGF) and BV8 are newly identified proteins described as selective angiogenic mitogens. We previously identified PK binding sites, two closely homologous G protein-coupled receptors (PK-R1 and PK-R2) in human and bovine ovarian cells, but their function remained unknown. In this study we examined the presence and effects of PK in CL-derived endothelial and steroidogenic cell types (LEC and LSC, respectively). PK-1 mRNA was identified in CL and follicles by real-time PCR, using primers specific for the bovine PK-1 sequence (retrieved from *Bos taurus* whole genome shotgun database). PK were potent angiogenic mitogens for LEC; they enhanced cell proliferation, elevated [^{3}H]thymidine incorporation, MAPK activation, and c-*jun/fos* mRNA expression. The effects of PK proteins on cell survival were examined by nuclear morphology (4′,6-diamidino-2-phenylindole dihydrochloride staining), measurement of DNA fragmentation (terminal dUTP nucleotide end labeling assay), and caspase-3 cleavage. Results obtained by these techniques demonstrated that PK protected LEC from serum starvation-induced apoptosis. Stress conditions such as serum withdrawal, TNF-α, and hypoxia markedly increased PK-R2 expression, whereas mRNA levels of PK-R1 remained unchanged. These suggest that the antiapoptotic effect of PK-1 on LEC may be mediated via PK-R2. PK-1 increased VEGF mRNA expression by LSC, implying that it could also indirectly, via VEGF, affect luteal angiogenesis. Together, these findings suggest an important role for PK-1 in luteal function by acting as a mitogen and survival factor in LEC. (*Endocrinology* 146: 3950–3958, 2005)

PROKINETICINS (PK) ARE a newly identified family of secreted proteins that possess diverse biological functions (1–8). In humans, they include PK-1, the homolog of mamba intestinal toxin 1 (MIT1), and PK-2, a homolog of *Bombina variegata* skin protein (Bv8). These factors share 60% amino acid identity and have common protein structural motifs of five disulfide bonds and identical amino-terminal sequences. Kaser *et al.* (9) proposed using their first four residues, AVIT, as a name to avoid the multiple names assigned to these proteins.

The PK are the cognate ligands for two closely homologous G protein-coupled receptors, PK receptors (PK-R1 and PK-R2) that share approximately 85% amino acid identity and are about 80% identical to a previously described mouse orphan receptor gpr73 (10–12). Expression of PK receptors in heterogeneous systems shows that these receptors bind and are activated by nanomolar concentrations of recombinant PK. Signaling via these receptors leads to calcium mobilization, stimulation of phosphoinositide turnover, and activation of the MAPK pathway (10–13).

PK-2 shows the highest expression in testis, brain, and peripheral blood leukocytes (3, 6, 14); within the central nervous system, it acts to increase pain sensitization and to activate the circadian clock (3, 4). PK-1 mRNA expression has been described in a variety of tissues, in steroidogenic glands (ovary, testis, placenta, and adrenal gland) but also in the gastrointestinal tract, nervous system, bladder, and prostate (1, 2, 11).

Originally identified as a potent agent in contracting smooth muscle of the gastrointestinal tract (1), PK-1 was consequently shown to act also as an angiogenic mitogen; intraovarian delivery of PK-1 promoted angiogenesis and cyst formation in the rat ovary (2). Additionally, it induced proliferation, migration, and fenestration of endothelial cells (EC) derived from adrenal capillaries but not of other endothelial-cell types such as those derived from the aorta or an umbilical vein (2). Therefore, it was termed endocrine gland-derived vascular endothelial growth factor (EG-

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Abbreviations: AP-1, Activating protein 1; BS-1, Bandeiraea simplicifolia lectin-1; DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride; DFX, deferoxamine mesylate; EC, endothelial cell; FBS, fetal bovine serum; GC, granulosa cell; G3PDH, glyceraldehyde 3-phosphate dehydrogenase; LEC, luteal endothelial cell; LSC, luteal steroidogenic cell; PK, prokineticin; PK-R, prokineticin receptor; PI, propidium iodide; TBST, Tris-buffered saline with Tween 20; TC, theca cell; TUNEL, terminal dUTP nucleotide end labeling; VEGF, vascular endothelial growth factor.

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VEGF), even though it is structurally unrelated to VEGF. PK-1 was identified in ovaries of several species, but its localization in the various cellular compartments of the ovary has been controversial; Ferrara and colleagues (2, 15) used *in situ* hybridization to localize it primarily to the theca layer and ovarian stroma in the human ovary, whereas other studies identified PK-1 mRNA in luteinized granulosa cells (GC) (16, 17). In studying the regulation of PK expression in human GC, we found that PK-1 was markedly augmented by forskolin (16). A similar induction of PK-1 was also observed in luteinized human GC incubated with human chorionic gonadotropin (17). In contrast to cAMP, classical proangiogenic cues, hypoxia and thrombin, inhibited PK-1 mRNA expression (16).

PK-1 was identified in the human corpus luteum (CL) (15, 17), but its effects on luteal cells remained unknown. Extensive angiogenesis takes place in the developing CL and results in an elaborate network of capillaries (18–21). These capillaries perfuse the CL and endow it with one of the highest blood flows per unit mass in the body, so that factors affecting vascular growth are likely to play a major role in regulating luteal function. One of the key factors in luteal angiogenesis is VEGF, which is expressed in the CL in a time-dependent manner (22, 23). Being a highly vascular endocrine gland (18, 21, 24, 25), the CL is an attractive model to study the effects of PK-1 on EC function. The presence of the two PK-R types in EC of the bovine CL (16) further support a role for PK in these cells. The present study was designed to determine whether PK are expressed in bovine ovaries and to examine their effects on the proliferation and survival of luteal EC (LEC).

Materials and Methods

Materials

Cell media, DMEM-F12 (1:1), high-glucose DMEM, Medium M-199, Hanks' balanced salts solution, L-glutamine, gentamycin sulfate, and crystalline trypsin solution 0.02%, were from Biological Industries (Kibbutz Beit Hemeek, Israel). Vitrogen type I collagen was from Cohesion Technologies (Palo Alto, CA). BSA, deferoxamine mesylate (DFX), cobalt chloride ($CoCl_2$), pertussis toxin, propidium iodide (PI), 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI), proteases inhibitor cocktail, acrylamide/bis-acrylamide electrophoresis reagent 40% solution, paraformaldehyde, and horseradish peroxidase-conjugated goat antirabbit IgG were from Sigma Chemical Co. (St. Louis, MO). *Bandeiraea simplicifolia* lectin-1 (BS-1) was from Vector Laboratories, Inc. (Burlingame, CA). Collagenase I, collagenase IV, hyaluronidase, and DNase I were obtained from Worthington Biochemical Corp. (Freehold, NJ). Uncoated magnetic beads (Dynabeads M450) were from Dynal (Oslo, Norway). [methyl-^{3}H]Thymidine and the prokaryotic expression vector pGEX-3X were purchased from Amersham Biosciences (Little Chalfont, UK). TriReagent was from MRC (Cincinnati, OH). Fetal bovine serum (FBS), SuperScript II RNase H$^-$ reverse transcriptase and Ultrapure electrophoresis agarose were obtained from Invitrogen Corp. (Paisley, UK). Random hexamer oligodeoxynucleotides were from Fermentas (Vilnius, Lithuania). Deoxynucleotide triphosphates and BioTaq DNA polymerase were obtained from Bioline GmbH (Luckenwalde, Germany). Oligo-dT and oligonucleotide primers were synthesized by MWG Biotech AG (Ebersberg, Germany). The real-time PCR SYBR Green Master-Mix Kit was from Eurogentec (Seraing, Belgium). Rabbit anti-p-44/42 MAPK were obtained from Cell Signaling Technology (Beverly, MA). Anti-cleaved caspase-3 and anti-total caspase-3 antibody were kindly provided by A. Gross of the Weizmann Institute of Science (Rehovot, Israel). VEGF was a generous gift from G. Neufeld of the Technion-Israel Institute of Technology (Haifa, Israel). Recombinant human TNF-α was obtained from ProSpec-TanyTechnoGene Ltd (Rehovot, Israel). The Mebstain Apoptosis Kit Direct was from Medical and Biological Laboratories Co. (Naka-ku Nagoya, Japan). SuperSignal West Pico chemiluminescent substrate was purchased from Pierce (Rockford, IL). The DC protein assay kit was from Bio-Rad Laboratories (Hercules, CA). X-ray films were from Fuji Photo Film Co., Ltd. (Tokyo, Japan).

Production of PK-1 and PK-2

Production, refolding, and purification of recombinant PK-1 and PK-2 were carried out appropriately as described previously (1). The activity of PK was further confirmed by calcium mobilization assay in CHO cells that stably expressed human PK-R1 or PK-R2 (10).

Isolation and culture of LEC

CL were collected at a local slaughterhouse and were confirmed to be at the midluteal stage (d 8–12) by macroscopic examination, according to criteria described by Fields and Fields (26). LEC were isolated and cultured as previously described (27, 28). Briefly, the CL were minced, and the fragments were passed through a series of sieves. The final cell pellet was resuspended in 50% isotonic Percoll and fractionated by centrifugation at 1600 × *g* for 20 min. The lower fraction, between the lower red blood cell band and the upper luteal cell band, enriched with vascular cells, was collected. Cells were cultured in DMEM-F12 (1:1) containing 10% FBS, 2 mM L-glutamine, and 50 μg/ml gentamycin sulfate, in culture plates precoated with 1% Vitrogen. Preliminary studies had shown that expression of PK-R and mitogenic response to PK remained similar at least until passage 12; therefore, cells from passages 4–12 were used for these experiments.

Enrichment of luteal steroidogenic cells (LSC) and EC

For enrichment of luteal cell subpopulations, mid-cycle CL were dispersed by means of collagenase IV as previously described (29, 30). Briefly, CL were sliced and dispersed in Medium 199 containing 0.5% BSA and collagenase IV (420 U/ml). Dispersed luteal cells were mixed with epoxy magnetic beads that had been precoated with BS-1. Both BS-1-positive cells (enriched endothelial cells) and nonadherent cells (enriched steroidogenic cells) were seeded and cultured overnight in DMEM-F12 containing 10% FBS, 2 mM L-glutamine, and 50 μg/ml gentamycin sulfate. Freshly isolated LEC were then incubated in DMEM-F12 with 0.5% BSA for 6 h, and PK (50 nM each) or VEGF (20 ng/ml) was added in the last 5 min. Proteins were extracted from the cells with SDS lysis buffer and were immunoblotted with p42/p44 MAPK antibodies as detailed below. LSC were incubated for 24 h in DMEM-F12 with 1% FBS only, and PK-1 (50 nM) with or without LH (100 ng/ml).

Isolation of granulosa and theca cells

Healthy (*i.e.* estradiol concentration in follicular fluids > 150 ng/ml) large bovine follicles were used. Granulosa and theca cells were enzymatically dispersed as previously described and were collected separately (31). Briefly, granulosa cells were aspirated with DMEM containing 0.1% hyaluronidase, 0.1% collagenase I, and 5 μg/ml DNase I. The theca interna layer was peeled from the theca externa with fine forceps and incubated in 0.25% trypsin/0.02% EDTA at 37 C for 15 min, followed by 45 min of incubation in DMEM with 3% collagenase I and 10 μg/ml DNase I.

RNA isolation and real-time PCR

Total RNA was isolated from the cells with TriReagent according to the manufacturer's instructions. PCR were performed using a GeneAmp 5700 sequence detection system (Applied Biosystems, Foster City, CA), with the SYBR Green I PCR kit used as described by Klipper *et al.* (32) but with several modifications. Briefly, each real-time reaction (18 μl) contained SYBR Green Master Mix that comprised ROX passive reference (200 μM dNTPs including dUTP, 5 mM $MgCl_2$, uracil *N*-glycosylase, and AmpliTaq HotGoldStar DNA polymerase), 0.54 μl of a 1:10,000 dilution of SYBR Green stock solution, 1.5 mM dNTPs, 10 nM of each primer, and 25–50 ng cDNA. Glyceraldehyde 3-phosphate dehydrogenase (G3PDH) gene was used as standard. A dissociation curve analysis

was run after each real-time experiment to confirm the presence of only one product and the absence of formation of primer dimmers. The threshold cycle number (Ct) for each tested gene X was used to quantify the relative abundance of the gene: $2^{-(Ct\text{gene X} - Ct\text{G3PDH})} \times 1000$. Table 1 presents a list of primers.

[^{3}H]Thymidine incorporation

Incorporation of [^{3}H]thymidine was measured according to Masuda *et al.* (11). Briefly, LEC were incubated for 28 h in 24-well plates with PK, VEGF, and DFX in DMEM containing 0.1% FBS and 0.5% BSA. [^{3}H]Thymidine (1 μCi/well) was added during the last 8 h of incubation. The cells were then washed with cold Hanks' balanced salts solution and incubated with 10% trichloroacetic acid at 4 C for 15 min. The acid-insoluble fraction was dissolved in 0.3 N NaOH (15 min at 37 C), and the radioactivity was evaluated with a scintillation counter.

Cell proliferation assay

LEC were plated at a density of 20,000 cells in 24-well plates. The cells were then cultured with one of the two PK proteins (50 nM) or VEGF (20 ng/ml) in DMEM-F12 containing 1% FBS. After 4 d of incubation, the medium was removed, the monolayers of cells were washed twice with PBS, and the cells were trypsinized. The cultures in each treatment group were assessed at least in triplicate; the cell numbers were determined with a hemocytometer and their viability by trypan blue dye exclusion.

Immuno (Western) blotting

Detection of p44/42 MAPK activation. Proteins were extracted from LEC with SDS lysis buffer [62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 50 mM dithiothreitol, and 0.01% bromophenol blue] and were sonicated for 10 sec. Protein extracts were separated on 12% SDS-polyacrylamide gel and transferred to nitrocellulose membranes. The membranes were blocked in Tris-buffered saline with Tween 20 (TBST) [20 mM Tris (pH 7.4), 150 mM NaCl, and 0.05%Tween 20] containing 5% nonfat milk for 1 h at room temperature and were incubated overnight with either phosphorylated p42/p44 MAPK or total p42/p44 MAPK antibodies diluted in TBST/5% BSA (1:10,000 and 1:1000, respectively) at 4 C. The membranes were washed and then incubated with horseradish peroxidase-conjugated goat antirabbit IgG diluted in TBST/5% nonfat milk at room temperature for 2 h. A chemiluminescent signal was generated with SuperSignal, and the membranes were exposed to x-ray film.

For detection of cleaved and noncleaved caspase-3, proteins were extracted from cells with 3-[(3-choloamidopropyl)-dimethylammonio]-1-propane-sulfate cell (CHAPS) extraction buffer [50 mM piperazine-1,4-bis (2-ethane sulfonic acid)/NaOH (pH 6.5), 2 mM EDTA, 0.1% CHAPS, 5 mM dithiothreitol, and protease inhibitor cocktail], and the protein contents of the cell lysates were determined with the DC protein assay kit. The extracted proteins were size-fractionated by 15% SDS-PAGE, transferred to nitrocellulose membranes, blocked in TBST/5% nonfat milk for 1 h, and then incubated overnight at 4 C, with anti-total caspase-3 and anti-cleaved caspase-3 antibodies. The antibodies were polyclonal anticaspase-3 diluted 1:1000 (this antibody recognizes full-length caspase-3 and weakly recognizes the p20 and p17 cleavage products) and polyclonal anti-cleaved caspase-3 diluted 1:300 (this antibody recognizes the p20 and p17 cleavage products of caspase-3 but not full-length caspase-3). Western blots were developed by use of the enhanced chemiluminescence signal generated as described above.

TABLE 1. Primers list

Gene	Sequence	Product length (bp)
β-Actin[a]	F: ccaaccgtgagaagatgaccca R: cgcagtccatttagaagcattt	800
PK-R1[a]	F: tgtggtatcggcaacttcat R: cccacgaactcgatgccaaa	505
PK-R2[a]	F: tataccccaagaacatgcct R: ggcccacgaattctatgcc	444
G3PDH	F: cgtggacagtggtcataagt R: ggcgtgaaccacgagaagtat	141
c-fos	F: gaatctgaggaggccttcacc R: tcagccttcagctccatgc	103
c-jun	F: agagcggcgcctacggctacag R: gtgaggaggtcggagttcttg	123
VEGF	F: ccatgaactttctgctctcttgg R: tccatgaacttcaccacttcg	135
PK-R1	F: ggtcatggatgagaatgcca R: atacccaccagcgccatgcc	200
PK-R2	F: ataccccaagaacatgcct R: ctgatacccacaagtgccacc	150
PK-1	F: ccacaaggtccccttcttcag R: gatgttcttcaagttggtggagc	121

F, Forward; R, reverse.
[a] Primers used in semiquantitative RT-PCR.

Detection of apoptosis

Quantification of apoptosis by nuclear morphology. LEC were grown for 48 h in DMEM-F12 containing 0.1% FBS, alone or with PK (50 nM), on coverslips that had been precoated with 1% Vitrogen up to 70–80% confluence. The cells were fixed with EFA (70% ethanol, 4% paraformaldehyde, 5% glacial acetic acid), permeabilized with 0.25% Triton X-100 in PBS, and then stained with 1 μg/ml DAPI reagent (33). The coverslips were mounted on glass slides and photographed under a fluorescence microscope. Apoptotic cells had condensed and fragmented nuclei (33). For the analyses, 11–15 fields of view at ×640 magnification were quantified (15–20 cells per field) in each experiment. The percentage of apoptotic cells in each field was evaluated, and the average over all fields was determined.

Quantification of apoptosis by terminal dUTP nucleotide end labeling (TUNEL). For the TUNEL assay, LEC were grown in 25-mm flasks that had been precoated with 1% Vitrogen up to 70–80% confluence and were cultured with PK as described above. Adherent cells were removed from the flasks with trypsin, combined with spent medium including floating cells and cellular debris, centrifuged, and then analyzed with the Mebstain direct apoptosis kit according to the manufacturer's recommendations. In brief, 0.5×10^6 LEC were fixed with 4% paraformaldehyde in PBS, permeabilized in 70% ethanol, washed in PBS, and incubated with staining solution (Tris buffer, terminal deoxynucleotidyl transferase enzyme, and fluorescein isothiocyanate-dUTP) for 1.5 h. The cells were counterstained with PI (4 μg/ml), and 20,000 cells were analyzed for both fluorescein isothiocyanate and PI fluorescence on a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ). Cells that had been incubated with staining solution without terminal deoxynucleotidyl transferase enzyme served as a negative control.

Statistical analyses

Data are presented as means ± SEM. The one-way ANOVA Tukey-Kramer test was used to determine the statistical difference between treatments, as indicated in the *Results* and the figure legends. Additionally, Student's *t* test was used to evaluate the effect of TNF-α on PK-R2 mRNA by LEC and PK-1 on the expression of VEGF by LSC. Differences were considered significant at $P < 0.05$.

Results

Expression of PK mRNA in bovine ovary

Initially, to determine whether PK were produced within the bovine ovary, we blasted *Bos taurus* whole genome shotgun sequences, with human PK-1 protein used as a query (AF333024.1 using tblastn tool at NCBI with WGS database). Two contigs that covered the 3′ and partial 5′ regions of the molecule (gi:53041764, AAFC01617108 and gi:52955958, AAFC01702914), respectively, were identified. The predicted partial protein sequence of bovine PK-1 (spanning the region of 25–105 amino acids; Fig. 1A) shared 88% identity with the corresponding human molecule (Fig. 1B). Two primers that overlapped bovine PK-1 by 121 bp were designed, and se-

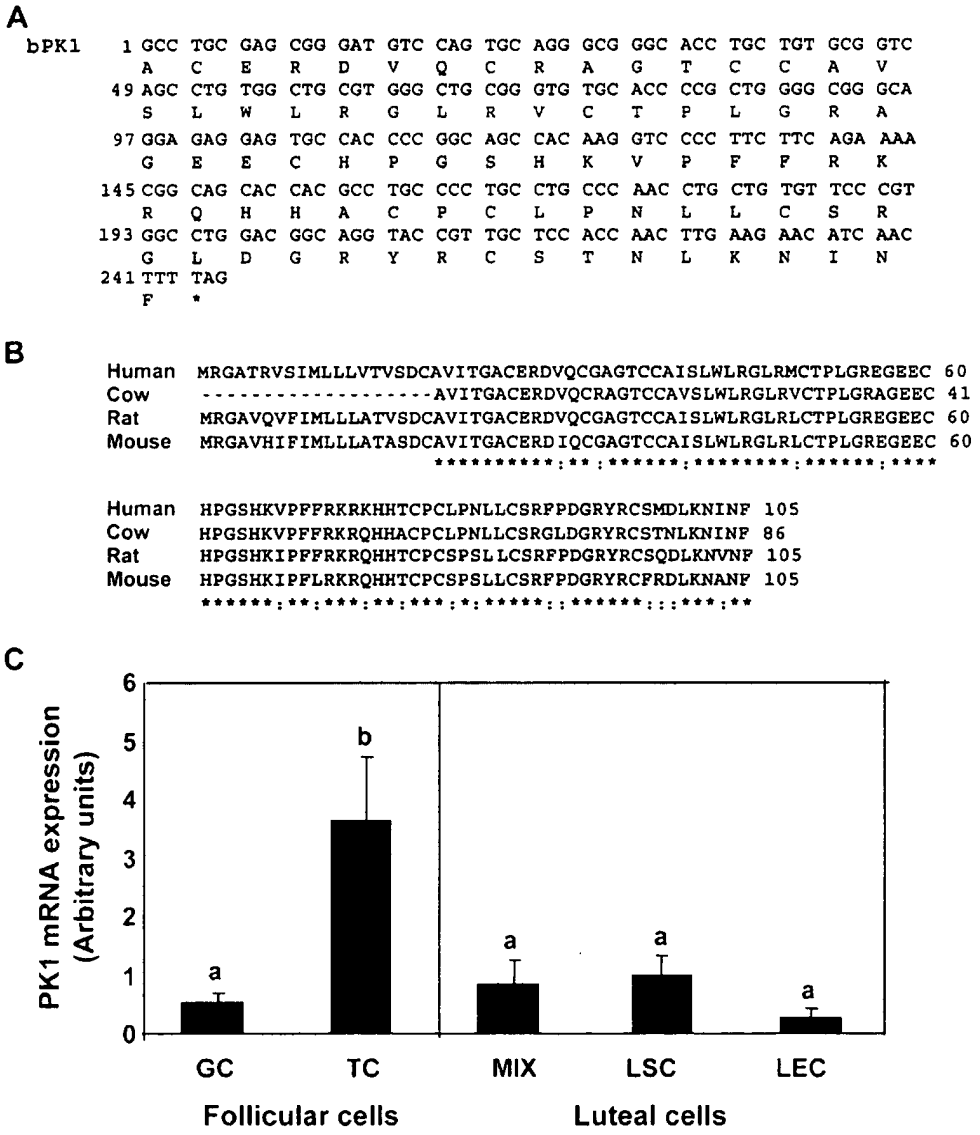

FIG. 1. Expression of PK-1 in bovine ovarian cells. A, Bovine PK-1 cDNA and amino acid partial sequences. B, Alignment of predicted bovine PK-1 amino acid sequence with human, rat, and mouse orthologs. *Asterisks* denote identity and *semicolons* mismatch in amino acid residues. C, Levels of PK-1 mRNA expression in bovine ovarian cell types as determined by real-time PCR. Follicular cells were GC (n = 15) and TC (n = 11). Luteal cells were a mix, total dispersed luteal (n = 14), freshly isolated LSC (n = 7), and EC (n = 6). *Different letters* (a and b) indicate significant difference between treatments ($P < 0.001$).

quence analysis ascertained the identity of the PCR product. This sequence was deposited in the GenBank and assigned the accession number AY877432. Real-time PCR conducted with these primers showed that steroidogenic bovine luteal cells and follicular GC expressed PK-1 mRNA at similar levels (Fig. 1C). In large follicles, significantly higher levels were present in theca cells (TC) than in the other steroidogenic cell types (GC and luteal cells). The residual expression of PK-1 mRNA in LEC was probably the result of contamination by steroidogenic cells as indicated by steroidogenic cell marker (steroidogenic acute regulatory protein) present in LEC (29). Bovine PK2 (accession no. NM_199445) was not detected in bovine ovary, but, as expected, it was readily detectable in bovine testis (data not shown).

Effect of PK on [^{3}H]thymidine incorporation and LEC proliferation

Proliferation of LEC was evaluated by [^{3}H]thymidine incorporation assays and cell counts (Fig. 2). PK-1 enhanced [^{3}H]thymidine incorporation in a dose-dependent manner, with maximum stimulation attained at a concentration of 100 nM (Fig. 2A). The two PK increased [^{3}H]thymidine incorporation by LEC to almost the same level as VEGF (by factors of ~1.6, 1.7, and 2 over control, for PK-1, PK-2, and VEGF, respectively; Fig. 2B). The cell counts were consistent with the results of the [^{3}H]thymidine incorporation assay; incubation with PK and VEGF increased LEC numbers 1.5-fold and 2-fold, respectively (Fig. 2C). Pertussis toxin (200 ng/ml)

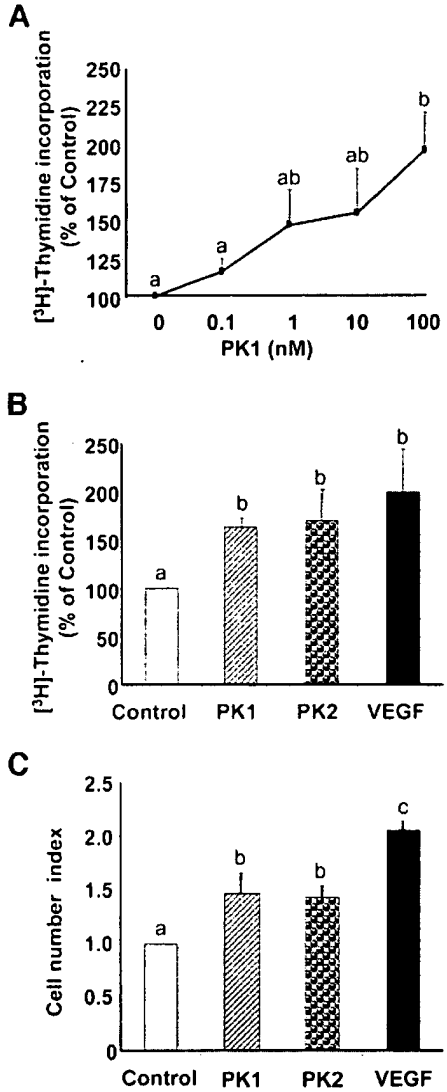

FIG. 2. Effects of PK on [^{3}H]thymidine incorporation and on proliferation of LEC. A, Dose-response curve of [^{3}H]thymidine incorporation in response to PK-1 (0–100 nM). B, Effects of PK (50 nM each, 28 h) and VEGF (20 ng/ml, 28 h) on [^{3}H]thymidine incorporation. C, Effects of PK (50 nM each) and VEGF (20 ng/ml) on LEC proliferation. Approximately 20,000 cells per well were seeded and incubated for 4 d in media containing 1% FBS. For each experiment, results were normalized to the respective control (71,500 ± 3753 cells per well). Data represent means ± SEM from three, six, and three experiments for data shown in panels A, B, and C, respectively. *Different letters* (a, b, and c) indicate significant difference among the treatments ($P < 0.05$).

did not significantly affect PK-1-induced [^{3}H]thymidine incorporation (89 ± 24% compared with PK-1 alone).

Hypoxia is a well characterized inducer of EC proliferation (34, 35); therefore we next examined the mitogenic effect of PK-1 on LEC that were exposed to the hypoxia-mimicking agent DFX (an iron chelator). As depicted in Fig. 3, almost a 2-fold increase in [^{3}H]thymidine incorporation was observed in cells incubated with DFX. Addition of PK-1 induced a 2.5-fold increase in [^{3}H]thymidine uptake in the presence of DFX, as compared with the control, whereas PK-1 alone induced a 1.7-fold increase.

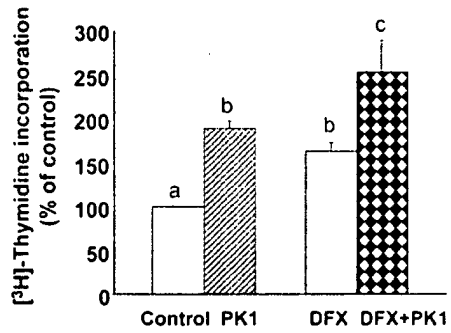

FIG. 3. Augmentation of [^{3}H]thymidine incorporation in LEC by PK-1, in the presence of hypoxia-mimicking agent DFX. Cells were incubated for 28 h with DFX (100 μM) in the presence or absence of PK-1 (50 nM). Within each experiment, results were normalized to control (100%). Data represent means ± SEM of three independent experiments for DFX in the presence or absence of PK-1 and of nine independent experiments for PK-1. Significantly different treatments ($P < 0.05$) are indicated by *different letters* (a, b, and c).

MAPK activation and induction of c-fos and c-jun mRNA expression in response to PK

The MAPK pathway is a well-recognized signaling mechanism that mediates mitotic processes in numerous mammalian cells (36). Having demonstrated that PK have mitogenic activity in LEC, we next examined whether PK activate p44/42 MAPK in these cells. We observed that both PK (50 mM, 5 min) induced the phosphorylation of p44/42 MAPK in freshly isolated LEC (Fig. 4), and, as expected, VEGF (20 ng/ml, 5 min) also induced the activation of p44/42 MAPK (Fig. 4).

The activating protein 1 (AP-1) family of transcription factors, *i.e.* the gene products of the c-*fos* and c-*jun* families of protooncogenes, play important roles in regulating gene expression (37, 38), and p44/42 MAPK is one of the many signaling pathways that are known to induce c-*fos* and c-*jun* expression (38, 39). To explore the role of AP-1 transcription factors in signaling by PK, we examined the induction of c-*fos* and c-*jun* mRNA expression in LEC. As shown in Fig. 5, PK-1 was a potent inducer of c-*fos* and c-*jun* mRNA expression; it transiently augmented c-*fos* mRNA expression in LEC with maximum stimulation (a 7-fold increase, attained at 1 h) (Fig. 5A), and it also increased c-*jun* mRNA expression, but to a lesser extent, *i.e.* a 2-fold increase at 1 h (Fig. 5B).

Protection of LEC from serum starvation-induced apoptosis by PK

The effect of the two PK on survival of LEC was examined by three different methods: nuclear morphology (DAPI staining), detection of DNA strand breaks (TUNEL assay),

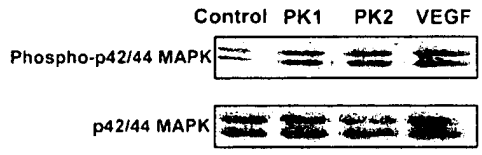

FIG. 4. Activation by PK of p42/p44 MAPK pathway in freshly isolated LEC. Cells were treated with PK (50 nM) or VEGF (20 ng/ml) for 5 min and then subjected to Western blot analysis with anti-phospho-/total-p42/44 MAPK. A representative blot (one of three similar ones) is shown.

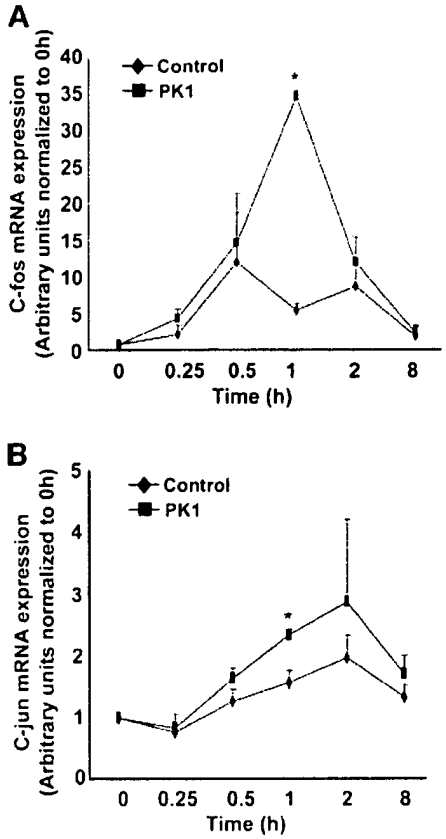

FIG. 5. Induction of c-*fos* and c-*jun* mRNA expression in LEC by PK-1. Time course of c-*fos* (A) and c-*jun* (B) mRNA expression in response to PK-1. LEC were incubated with basal media only (0.1% FBS, 0.5% BSA in DMEM-F12; control) or media containing 50 nM PK-1, for up to 8 h. At each time point, RNA was extracted and mRNA levels were determined by real-time PCR. Within each experiment, results were normalized to 0 h (n = 3). *, Significant difference ($P < 0.05$), as compared with the control at the corresponding time point.

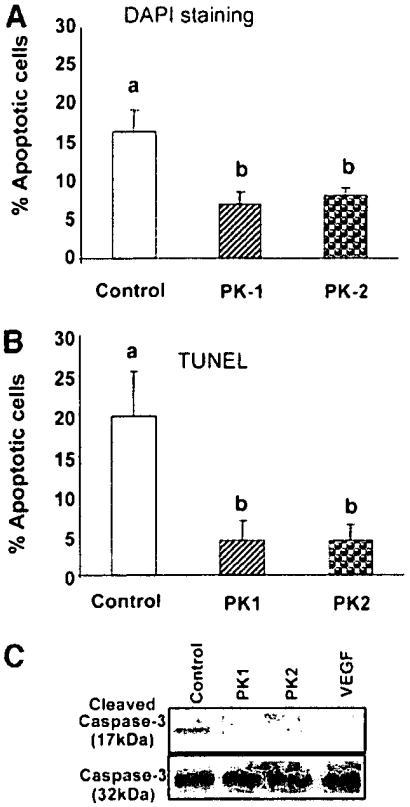

FIG. 6. Inhibition of serum starvation-induced apoptosis in LEC by PK. Cells were grown for 48 h in medium containing either 0.1% FBS alone, PK (50 nM), or VEGF (20 ng/ml). A, DAPI staining. The percentage of apoptotic nuclei was evaluated as described in *Materials and Methods*. B, TUNEL assay. The percentage of TUNEL-positive, apoptotic cells was evaluated by flow cytometry. Data (for A and B) are means ± SEM from three separate experiments. *Different letters* (a and b) within each graph indicate significant difference among treatments ($P < 0.05$). C, Cleaved and total caspase-3 were determined by Western blot analysis with specific antibodies. A representative blot is presented.

and induction of the apoptotic cascade (cleavage of caspase-3). DAPI staining of apoptotic nuclei revealed 16.5 ± 2.8% of the nuclei in serum-starved cells to be apoptotic, whereas the presence of PK-1 or PK-2 significantly decreased this proportion to 7.0 ± 1.5 or 8.0 ± 1.2%, respectively (Fig. 6A).

Serum starvation of LEC typically resulted in 19.9 ± 5.6% of cells becoming apoptotic as assessed by flow cytometry detection of TUNEL-positive cells (Fig. 6B). PK-1 and -2 were able to rescue the cells and significantly reduced the apoptosis rate to 4.3 ± 2.3 and 4.3 ± 1.9%, respectively (Fig. 6B).

Activation of caspase-3 requires proteolytic processing of inactive zymogen (32 kDa) (40). The amount of cleaved caspase-3 was determined by Western blot analysis with a specific antibody (17 and 19 kDa). In accordance with data shown in Fig. 6, A and B, both PK, similarly to VEGF, completely inhibited the serum starvation-induced cleavage of caspase-3 (Fig. 6C).

Regulation of mRNA expression of PK receptors under stress conditions

Because PK act as survival factors for LEC, it was of interest to examine whether the expression of PK receptors was modulated by various stress factors such as serum starvation, TNF-α, and hypoxia induced by DFX or $CoCl_2$. All treatments significantly elevated PK-R2 mRNA. A gradual reduction of serum concentrations in the culture medium, from 10 to 0.1% increased PK-R2 mRNA expression markedly (3.8-fold), whereas the PK-R1 mRNA levels remained unchanged (Fig. 7A). Likewise, exposure of LEC to the hypoxia-mimicking agents DFX and $CoCl_2$ or to the cytokine TNF-α also augmented PK-R2 mRNA expression. In fact, the levels of PK-R2 were elevated beyond those found after serum withdrawal only (controls, 0.1% FBS; Fig. 7B). Unlike PK-R2, PK-R1 levels were not significantly affected by stress conditions, besides a reduction observed in cells exposed to $CoCl_2$ (Fig. 7B). For the last set of data we used β-actin as a housekeeping gene, because hypoxia modulates the expression of G3PDH mRNA (Table 1).

Augmentation of VEGF mRNA expression in LSC by PK-1

In light of our previous finding that LSC also express PK-R1 (16), we next examined whether PK-1 might affect the functions of LSC. As depicted in Fig. 8, PK-1 significantly elevated the

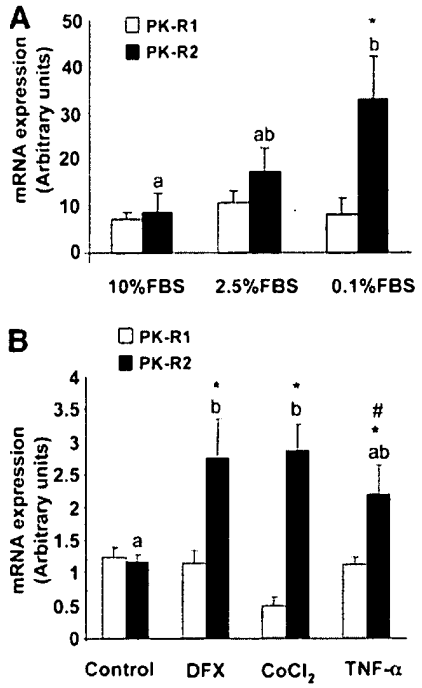

FIG. 7. Modulation of PK-R expression by stress conditions in LEC. A, Effect of serum withdrawal on PK-R expression. Cells were incubated in medium containing various concentrations of FBS (10, 2.5, and 0.1%) for 48 h. PK-R1 and PK-R2 mRNA expression were determined by real-time PCR. Data are mean ± SEM of three separate experiments. B, Effect of hypoxia-mimicking agents (DFX and $CoCl_2$) and TNF-α on PK-R expression in LEC. The cells were incubated in basal media (0.1% FBS, 0.5% BSA in DMEM-F12) only (control) or with 100 μM DFX, 100 μM $CoCl_2$, and 10 ng/ml TNF-α for 16–48 h. The mRNA levels of PK-R1 and PK-R2 were determined by semiquantitative RT-PCR using β-actin as internal standard. Data are the densitometric units of PK-R1 and PK-R2 relative to β-actin from five independent experiments. *Different letters* (a and b) within each graph show where differences in PK-R2 expression are significant ($P < 0.05$). *, Significant difference ($P < 0.05$) between PK-R1 and PK-R2 mRNA levels within the same treatment; #, significant difference ($P < 0.03$) in PK-R2 expression from control.

mRNA levels of VEGF by LSC. These effects were evident both in the presence and in the absence of LH (Fig. 8).

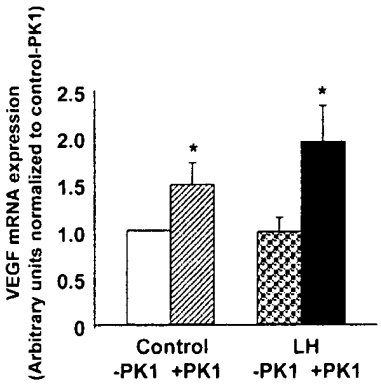

FIG. 8. Induction of VEGF mRNA expression in LSC by PK-1. LSC were obtained from mid-cycle CL as described in *Materials and Methods*. Cells were incubated with 50 nM PK-1 in the presence or absence of LH (100 ng/ml) for 24 h. VEGF mRNA expression was determined by real-time PCR. Within each experiment, results were normalized to control. Data are the mean ± SEM of five separate experiments. *, $P < 0.05$ denotes a significant difference from the respective control (either with or without LH).

Discussion

Evidence obtained in this study suggests that PK-1 may serve as a paracrine regulator in bovine ovary; the mRNA of the ligand (PK-1) and two receptors (PK-R1 and PK-R2) were identified in several ovarian cell types (present study and Ref. 16). Furthermore, PK elicit cell-specific effects in LSC (induction of VEGF) and in endothelial cells, where they acted as mitogenic and antiapoptotic factors. Finally, we showed that PK-R2 expression in luteal EC is dynamically regulated in response to stress conditions.

PK-1 mRNA was identified in bovine follicular and luteal cells, but the levels of PK-1 tended to be higher in follicular and luteal cells than in LEC, suggesting that the message is expressed mainly by steroidogenic cells. This conclusion is supported by previous studies that employed *in situ* hybridization of human ovaries (15), but it is still not clear whether PK-1 is expressed more in the theca or the granulosa compartments of follicles and CL (15–17). In the present study, significantly higher levels of PK-1 were found in bovine TC than GC, but it is still unclear whether this distribution is retained after luteinization, when the cells differentiate into luteal cells. Nevertheless, the present findings imply that both follicles and CL are important sources of ovarian PK-1. High PK-1 levels in the TC could be related to elevated LH receptor levels and cAMP responses in these cells (41, 42), because cAMP was shown to be a potent stimulator of PK-1 mRNA (16, 17). Additional work is necessary to unravel other factors that are likely to be involved in regulating PK-1 expression in TC. Initial studies have shown that both PK-1 and PK-2 are capable of activating PK-R in overexpressing cells, and it was similarly found in the present study with LEC that the effects of PK-1 and PK-2 overlapped. However, we could not detect PK-2 message in bovine ovarian tissue, nor was it detected in the human ovary by other workers (15, 17), so that the physiological source of the PK-2 in the ovary is presently unknown.

The CL is a short-lived endocrine gland that develops from the preovulatory follicle (43). During CL formation, thecal microvessels invade the granulosa cell layer and extensive angiogenesis ensues. This robust angiogenesis results in a dense vascular network at mid-cycle (18–20, 24). Therefore, the CL provides a unique model system for the study of the cellular and molecular regulation of physiological angiogenesis. Several lines of evidence described in the present paper indicate that PK are potent angiogenic mitogens for LEC; PK enhanced [^{3}H]thymidine incorporation, induced activation of MAPK, increased c-*jun*/c-*fos* mRNA expression, and induced cell proliferation.

The MAPK pathway is critical for cellular proliferation; MAPK activation promotes transcription of the cyclin D1 gene, which is rate limiting and essential for progression through the G1 phase of the cell cycle (37, 44). The MAPK pathway is also necessary for the association between cyclin D1 and Cdk4 (45). MAPK is one of the signaling pathways known to induce expression of the oncoproteins c-*fos* and c-*jun*, which belong to a class of immediate early genes that are rapidly activated, usually in a transient fashion, in response to intracellular signaling cascades. Fos and Jun contain a bZIP region consisting of a basic

DNA-binding domain and a leucine zipper domain, and together they form dimeric complexes that stimulate transcription of genes containing AP-1 regulatory elements (37, 38). AP-1 proteins bind directly to the cyclin D1 promoter and activate its transcription (38). The present study found that PK-1 rapidly and transiently stimulated the expression of c-*fos* and c-*jun* in bovine LEC. A similar, MAPK-dependent induction of c-*fos* and c-*jun* was demonstrated by Chen and Davis (39) in bovine luteal cells in response to epidermal growth factor.

Pretreatment of LEC with pertussis toxin, which specifically modifies the heterotrimeric G protein $G\alpha_i$ did not inhibit the effect of PK-1 on [^{3}H]thymidine incorporation, which indicates that $G\alpha_i$ may not be involved in PK-R activation in LEC. These results are in agreement with those previously reported by Lin *et al.* (10) and Soga *et al.* (12) but contradict those published by the group led by Ferarra in adrenal gland EC (13). The reason for this discrepancy remains unclear.

The present findings suggest that, in addition to activating LEC proliferation, PK efficiently inhibited cell death in these cells, as indicated by inhibition of DNA fragmentation and caspase activation. This indicates that PK are not only mitogens but also are survival factors for LEC. Caspases are a family of intracellular cysteine proteases whose actions are linked to both the initial and the final stages of apoptosis in virtually all types of vertebrate cells (46). Two general intracellular pathways that lead to apoptosis and that involve the activation of caspases exist in cells; the extrinsic pathway (caspase-8 and -10) involves activation of the TNF/Fas death receptor family, and the intrinsic pathway (caspases-9 and -2) acts through release of cytochrome *c* from mitochondria (40, 46). Caspase-3 is a key mediator of apoptosis, being either partially or totally responsible for the proteolytic cleavage of many key proteins, such as nuclear enzyme poly(ADP-ribose) polymerase (47). Caspase-3 was found to be a pivotal mediator of apoptosis in luteal cells during regression of the CL (48).

Importantly, PK were found to promote DNA synthesis in adrenal cortex EC (13), as they do in LEC, and these cells have much in common; irrigating endocrine glands, the fenestrated microvascular EC are particularly permeable to the inflow of blood-borne substances and the outflow of specific secreted products. It is noteworthy that adrenal cortex and LEC, unlike the EC derived from the bovine aorta, express the two PK-R types -R1 and -R2 equally (11, 16). Up to now, PK-R2 were only identified in fenestrated EC, such as those found in the adrenal cortex, CL, kidney, and liver (11, 16, 49), which suggests that PK-R2 may confer the selective effects of PK-1 on microvascular EC functions.

However, unlike the adrenal cortex, the CL is a short-lived gland in which a new vascular bed is developed during each cycle. This may suggest that the universal function of PK-1 in microvascular EC is to support cell viability (survival) and, possibly, also to maintain permeability. The pattern of PK-1 expression in the human CL, in which it peaks from the mid- to the late-luteal stage (17), also tends to confirm its role as a survival factor. However, because PK-1 is a secreted protein, the ligand necessary for angiogenesis in the CL could come from the developing first-wave follicle, which is present at this stage of the bovine cycle. Nevertheless, in the absence of sufficient data, this question requires further investigation.

Proper vascularization is essential for normal CL function (21, 50, 51); therefore, it is to be expected that angiogenesis-promoting agents such as PK-1 would play a major role in the regulation of luteal function. Likewise, follicular growth and the selection of dominant follicles also depend on the appropriate development of the microvascular bed in the thecal layer (52, 53). The PK-1 may thus contribute to the endocrine functions of the CL and follicles by inducing EC proliferation and permeability and thereby accelerating the transport of hormones and nutrients. Being a multifunctional factor, PK-1 is likely to have other roles in the ovary. Indeed, we found that in addition to its effects on EC, PK-1 also affected LSC function; it increased VEGF mRNA expression, possibly by acting via the PK-R1 present in these cells. VEGF has long been recognized as a key factor in angiogenesis in general and in the CL in particular (21, 25, 50, 54), and the present findings indicate that PK could also affect luteal angiogenesis indirectly, via VEGF. Thus, rising PK-1 levels from the mid to the late stages of human CL development could sustain the LEC at a time when the luteal VEGF levels begin to decline (17, 23). The presence of several EC-specific growth factors that maintain EC function and survival during the various developmental stages is essential for a tissue such as the CL.

In the present study, stress-inducing conditions such as serum withdrawal or addition of TNF-α or the hypoxia-mimicking agents increased PK-R2 expression in LEC. In contrast, the mRNA levels of PK-R1 and VEGFR-2 (data not shown) were not elevated or even reduced (in the presence of $CoCl_2$). These findings may imply that the antiapoptotic effect of PK-1 on LEC could be mediated via PK-R2, although a permissive role of PK-R1 cannot be ruled out. Up-regulation of PK-R2 expression under stress conditions could facilitate the cell response to PK-1 by triggering cell survival signaling cascades downstream. Additional studies, which would address the question of gain or loss of function, will be required to evaluate this hypothesis. Collectively, these findings suggest that PK-1 plays an important role in luteal function by promoting the proliferation and survival of CL-derived EC.

Acknowledgments

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